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## **Dynamics of neutrophil gelatinase-associated lipocalin plasma and cerebrospinal fluid concentrations in older males**

Petrus J.W. Naudé<sup>a,b\*</sup>, Doortje W. Dekens<sup>a,b\*</sup>, Ulrich L.M. Eisel<sup>b,c</sup>, Izaak den Daas<sup>d</sup>, Peter P. De Deyn<sup>a,e</sup>

<sup>a</sup>Department of Neurology and Alzheimer Research Center, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands. <sup>b</sup>Department of Molecular Neurobiology, Groningen Institute for Evolutionary Life Sciences, University of Groningen, Groningen, The Netherlands. <sup>c</sup>University Center of Psychiatry & Interdisciplinary Center of Psychopathology of Emotion Regulation, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands. <sup>d</sup>QPS Netherlands BV, Groningen, The Netherlands. <sup>e</sup>Laboratory of Neurochemistry and Behaviour, Institute Born-Bunge, University of Antwerp, Antwerp, Belgium.

\*Authors contributed equally to this manuscript

Corresponding author:

P.J.W. Naudé, Department of Molecular Neurobiology, Groningen Institute for Evolutionary Life Sciences, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands. p.j.w.naude@umcg.nl, Tel. +31 50 363 2363, Fax. +31 50 363 2331 .

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## **Abstract**

### *Background*

Neutrophil Gelatinase-Associated Lipocalin (NGAL) is an inflammatory protein with gaining increasing interest for its use as marker in blood and cerebrospinal fluid (CSF) for several chronic diseases. Its biochemical properties make it an attractive marker. However, changes in blood and CSF NGAL concentrations during the diurnal rhythm in the elderly are unknown. This information is important for its optimal use as marker in studies with older people.

### *Methods*

Serial paired plasma and CSF samples were obtained from 8 healthy elderly males over a 30 hour period. NGAL and cortisol were quantified with ELISA.

### *Results*

No significant changes in plasma and CSF NGAL concentrations over time were found, whereas cortisol (included as internal control) concentrations displayed significant changes over time. Significant circadian patterns were found for plasma NGAL and for cortisol in both plasma and CSF. However, CSF NGAL concentrations did not follow a diurnal pattern in elderly males.

### *Conclusions*

This study illustrates the temporal regulation of NGAL in plasma and CSF, which potentially is a useful reference for studies measuring NGAL as biomarker in older individuals.

Key words: Lipocalin 2, cortisol, repeated measures, cerebrospinal fluid, circadian rhythm.

## Introduction

Neutrophil Gelatinase-Associated Lipocalin (NGAL), also known as Lipocalin-2, siderocalin, 24p3, or uterocalin, is a 25 kDa acute phase inflammatory protein. NGAL exists as a monomer, homodimer or heterodimers with matrix metalloproteinase-9 (MMP9) [1]. It functions as a bacteriostatic agent by binding to bacterial siderophores and interferes with siderophore-mediated iron acquisition [2]. NGAL is gaining mounting interest in basic and clinical studies for its use as an inflammatory marker for various diseases. The associations of serum and plasma NGAL concentrations with disease are actively studied in various research fields, including cardiovascular disease [3], cancer [4] and neuropsychiatry i.e. depression [5]. Urine NGAL concentrations are studied in kidney injury [6, 7] and CSF NGAL concentrations are measured in multiple sclerosis [8], mild cognitive disorders and Alzheimer's disease [9]. In addition to the robust changes of NGAL concentrations in certain diseases, its attractiveness for studies rests upon its physical properties that make it suitable for application as a biomarker. It is resistant to proteolytic degradation [10] and possesses great storage stability [11, 12]. However, NGAL concentrations like other inflammatory markers oscillate according to a day/night cycle. A study by Scheer and colleagues in young healthy male participants showed that serum NGAL concentrations significantly change during a 24 hour day/night cycle [13]. However, aging can have a dampening effect on circadian rhythms [14]. In this respect, an altered circadian regulation of immune markers was found in elderly people [15]. However, the effect of an aging physiological environment on the diurnal regulation of NGAL concentrations in blood and CSF is still unknown. This is of importance for studying NGAL concentrations in clinical studies because the majority of research on this topic investigates the association of blood and CSF NGAL concentrations with diseases that primarily occur in older persons.

The main aim of this study is to characterize if the dynamics of NGAL in serial measures of plasma and CSF is reduced in older male volunteers by 1) investigating changes of concentrations over time, 2) determining circadian regulation of NGAL and 3) studying the relationship between plasma and CSF NGAL over time, with cortisol as reference marker.

## **Materials and methods**

### *Study volunteers*

After a screening process as previously described [16], serial CSF and plasma was obtained from eight healthy older volunteers of on average 63.25 years of age (Table 1). Study participants did not have any chronic or acute diseases and were not using any medication. The study protocol was approved by the local Institutional Ethics Committee (Stichting Beoordeling Ethiek Bio-Medisch Onderzoek, Assen, The Netherlands) and participants gave informed consent.

### *Sampling of plasma and CSF*

The methodology of the serial plasma and CSF sampling used in this study was described previously [16]. In brief, after being assessed for eligibility during a 3-week screening period, the subjects came to the Clinical Pharmacology Unit on the evening of Day -2. On Day -1 an intravenous cannula was inserted. During Day -1, each volunteer received 2 L of 0.9 % saline solution by infusion.

Intradural catheterization was performed on Day 1 by an experienced anesthesiologist in strict surgical aseptic conditions in a separate room. For antithrombotic purposes Fraxiparine (nadroparine calcium) was given 12 and 36 h after intradural catheterization and each subject wore elastic compression stockings.

Lights were turned off from 24h00 to 7h00 to allow participants to sleep. Subjects were allowed to eat before entering the study center. Meals were not to be taken within 1 h before start of procedure of inserting the intradural cannula. During the day in the study center, subjects received standard meals according to QPS standard operating procedures during their stay in the clinic. Drinking of alcoholic beverages was not permitted from day -2 until follow up. The intake of water, caffeinated coffee and tea was ad libitum during the whole study. Whilst resident, subjects were encouraged to drink at least 1,500mL fluid per day. Smoking was not permitted on the days of CSF sampling.

Paired plasma and CSF samples were collected (2 mL per sample) by interval sampling over a 30-h period on Day 1 and 2 under strict surgical aseptic conditions by the medical staff at the bed site. Samples were withdrawn at 1-hour intervals by aspiration with a syringe from  $t=0$  h to  $t=12$  h, at 4-h intervals between  $t=12$  h and  $t=24$  h and at 2-h intervals from  $t=24$  h to  $t=30$  h. This resulted in a total of nineteen paired serial samples. CSF and plasma serial sampling commenced at  $t=0$  (10h00) in the morning, immediately after fixation of the intradural catheter.

#### *Analyses of blood and CSF markers*

Plasma and CSF NGAL concentrations were quantified using human NGAL capture antibody (R&D Systems), recombinant human NGAL (R&D Systems) for the internal standard and biotinylated human NGAL detection antibody (R&D Systems) according to the manufacturer's protocol. Plasma was diluted 1:100 and CSF was not diluted for analyses. The intra- and inter-assay coefficients of variation were 3% and 5%, respectively. Cortisol in plasma and CSF and plasma C-reactive protein were

measured with ELISA (Abnova), according to the manufacturer's recommendations. The intra-assay coefficients of variation for cortisol and CRP were 3% and inter-assay coefficients were 9% and 10% respectively. Reporting of the study conforms to STROBE statement along with references to STROBE statement and the broader EQUATOR guidelines [17].

### *Statistical analyses*

A paired samples *t*-test on Ln-transformed CRP values was used to compare CRP concentrations between baseline measurement and the last measurement. Due to sampling at unequal intervals, a linear mixed model was used to determine changes of NGAL and cortisol over time. The Akaike information criteria (AIC) value indicated first-order autoregressive covariance structure as a better fit than compound symmetry, compound symmetry heterogeneous or Toeplitz. Therefore, first-order autoregressive covariance structure was used to account for the longitudinal relationship of samples. Time was included as fixed effect and subject as random intercept. NGAL and cortisol concentrations were log transformed prior to the analysis, which reduced skewedness and satisfied assumptions for homogeneity of variance. Covariates were not included due to the homogenous study group used in this study.

Rhythmic diurnal variations were determined with cosinor analyses [18]. Analysis was performed similar to that previously described [19]. Hourly concentrations of NGAL and cortisol in plasma and CSF of each participant were normalized to their respective mean concentration over 30 hours. The linear concentration rise over time for plasma and CSF NGAL and cortisol was subtracted out of the mean concentrations and a single cosinor fit was applied. A cosine transformation was applied to the time variable using

24 hours as the default circadian cycle. The amplitude, which is the distance between the peak and the midline of the cosine wave, was determined for NGAL and cortisol. Midline estimating statistic of rhythm (MESOR) is half of the midway between the highest and the lowest values of the fitted cosine curve. The acrophase represents the peak time of the fitted cosine. A 24-h rhythm was accepted if the null amplitude hypothesis was rejected ( $p < .05$ ) from an  $F$ -test. GraphPad Prism version 5.01 for Windows was used for analyses of diurnal rhythms of NGAL and cortisol in plasma and CSF.

Statistical analyses were performed with SPSS version 22. P-values were considered statistically significant at a value of less than .05.

## **Results**

### *Participants*

This study included healthy older men. The youngest subject was 57 and the oldest was 69 years of age. The mean BMI was  $24.0 \pm 2.0$  kg/m<sup>2</sup>, mean height was  $181 \pm 9.4$  cm and mean weight was  $78.9 \pm 8.3$  kg (Table 1). Plasma CRP concentrations were within normal range ( $<10$  mg/L), indicating the absence of acute inflammation [20]. Plasma CRP concentrations were not significantly different ( $p = .56$ ) between baseline (10h00, day 1) and last measurement (16h00, day 2) of the study. One participant withdrew from the study after the fourth hour due to difficulties in obtaining CSF samples. Because only four consecutive plasma samples and two CSF samples were obtained, this participant was excluded from further analyses.



### *NGAL and cortisol concentrations in plasma and CSF*

A mean peak value of  $139 \pm 45$  ng/ml for plasma NGAL was observed at 02h00 (Fig. 1A) and for CSF  $1229 \pm 281$  pg/ml at 06h00 (Fig. 1B). Repeated measures analysis with linear mixed model showed no significant changes in plasma NGAL concentrations ( $p=.26$ ) or NGAL concentrations in CSF ( $p=.17$ ) over time.

For plasma cortisol, a peak of  $243 \pm 115$  ng/ml was observed at 06h00 (Fig. 1C) and for CSF cortisol  $39 \pm 6$  ng/ml at 10h00 in the baseline measurement (Fig. 1D). As expected, significant changes of cortisol in plasma ( $p=.001$ ) and in CSF ( $p<.001$ ) over time were found.

### *Diurnal characteristics of NGAL in older participants*

Fig. 2 shows the cosine transformation of the mean-adjusted group average (percentage of mean) data for 30-h variations of NGAL and cortisol in plasma and CSF in older male subjects. For plasma NGAL, cosine analyses revealed a significant rhythm ( $p=.001$ ) with a peak time at 02h12 (Fig. 2A). NGAL concentrations in CSF, however did not follow a cosinor rhythm ( $p=.18$ ) (Fig. 2B). A significant cosinor fit was found for cortisol in plasma ( $p=.001$ ) (Fig. 2C) as well as for cortisol in CSF ( $p<.001$ ) (Fig. 2 D). Amplitude peaks for cortisol occurred at 07h10 in plasma and at 10h10 in CSF.

## **Discussion**

The main findings of this study show that the average plasma and CSF NGAL concentrations do not significantly change over time in older males. Plasma NGAL concentrations have a diminished circadian rhythm compared to plasma cortisol and no circadian rhythmicity was found for CSF NGAL concentrations in older individuals.

### *NGAL in plasma and CSF*

Our data shows that plasma NGAL concentrations significantly fit a cosinor circadian rhythm, which is in accordance with a previous study that reported a diurnal rhythm of NGAL concentrations in blood [13]. However, no significant changes in concentrations over time were found with mixed linear regression analyses. The reduced amplitude of the circadian rhythm for plasma NGAL shown in this study may explain less variation of plasma NGAL concentrations over time. In the study by Scheer *et al.*, young adults demonstrated 1) a higher peak-to-trough difference (40.4%) compared to the older persons (20.5%) examined in our study and 2) a different timing in peak and trough concentrations at 18h00 and 03h45, while we found peak and trough NGAL concentrations at 02h12 and 15h45, and 3) a lower 24-h mean NGAL level (29 ng/mL) compared to our study (116 ng/mL). Reduction in amplitude and changes in phase of circadian rhythm of blood NGAL concentrations might largely be caused by aging [14]. Higher serum and plasma NGAL concentrations are associated with increase of age [5, 21], which can explain the higher NGAL concentrations in the older people of this study. Of note, CRP concentrations were determined in the first and last plasma measures to evaluate inflammatory status of the study volunteers.

The current study is the first to analyze diurnal variations in CSF NGAL concentrations. Interestingly, our results show non-significance for cosinor fit and mixed linear regression for CSF NGAL concentrations. Considering single NGAL and cortisol CSF measurements in previous studies, it seems that the average CSF NGAL concentrations over time (1086 pg/mL) found in our study are in range with previous findings in healthy control subjects (although a wide range should be noted; [8, 9, 22, 23]), while the observed mean CSF cortisol level (27 ng/mL) may be on the high end as

compared to previous reports [24-26]. Yet it must be noted that the time of day at which samples in the previous studies were obtained often remains unknown, which makes it difficult to fully compare findings.

### *Cortisol in plasma and CSF*

Cortisol concentrations significantly changed over time and had a more robust circadian rhythm than NGAL in plasma and CSF. Our results indicate that the average blood cortisol concentrations were higher (131 ng/mL vs. 76 ng/mL), and the amplitude of the cortisol diurnal cycle was smaller (77.2% vs. 126.3%), compared to Scheer *et al.* (2010) as well as other studies (summarized by [27]). The higher mean plasma concentrations and smaller amplitude for plasma cortisol cycle in our study as compared to abovementioned studies can be explained by the older age of the study subjects. The elderly participants in the current investigation were on average  $63.3 \pm 5.0$  years of age, whereas the participants in studies of Scheer *et al.* and Debono *et al.* were much younger, with on average  $20.9 \pm 2.1$  years of age in the study of Scheer *et al.* [13, 27]. As shown in multiple studies, mean blood cortisol concentrations rise with age, and the amplitude of the cortisol cycle becomes smaller, which corresponds to our findings [15, 28-30].

Serial CSF analyses for cortisol have to our knowledge only been performed in non-human primates [31, 32]. Contrary to NGAL, cortisol concentrations in CSF significantly changed over time and displayed a significant circadian rhythmicity with a trough at 24h00 and peak at 10h10.

### *Utilization of NGAL as marker*

Results from this study indicate that the average NGAL concentrations are particularly stable between 10:00 until 21:00, which is a period when blood samples most likely will be collected in practice. Our data further shows that CSF NGAL concentrations remain stable over time in older individuals, suggesting that it is reliable marker compared to large variations observed for other inflammatory markers in CSF in serial sampling from older humans [33].

### *Study limitations*

Methodological issues warrant attention for proper interpretation. This study included a relatively small group size with only male participants. However, our group size compares with other studies in this line of research. Outcomes for our cosinor analyses may be skewed due to less frequent sampling between 22:00 and 10:00, which can explain some differences observed in the diurnal rhythm for cortisol found in this study compared to previously discussed literature. Strengths include the investigation of an elderly study group, high frequency sampling of paired blood and CSF samples over a period of 30 hours, and inclusion of cortisol as a reference marker. Although CSF sampling may be invasive, all participants evaluated their discomfort as no to moderate discomfort, and indicated that they would participate in a similar study again [16].

### **Conclusion**

This study confirms our hypothesis that diurnal variation in plasma and CSF NGAL levels are reduced in older males, compared to cortisol as reference marker. Findings from this study indicate that NGAL in plasma and CSF is an attractive marker due to its stability over time. The application of CSF NGAL concentrations as marker is

of particular interest for neuroscience related research due to its stability over time. Future studies are required to determine if a reduced diurnal regulation of NGAL is similarly present in older females. The effect of age-associated diseases on the diurnal regulation of NGAL should be verified for its accurate application as biomarker.

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### **Conflict of interest**

The authors herein declare no conflict of interest

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**Figure 1.** Mean plasma and CSF concentrations of NGAL and cortisol over time, presented as  $\pm$  standard error of the mean (SEM).

**Figure 2.** Diurnal regulation of plasma and CSF of NGAL and cortisol illustrated by cosinor fits. Data presented as mean-adjusted average of NGAL and cortisol levels over 30-hours for all subjects. (A) Plasma NGAL: amplitude, -10.76 (95% CI, -15.77 to -5.75); mesor, 103.5; acrophase, 02h12 (95% CI: 92.15 to 92.89). (B) CSF NGAL: amplitude, 3.1 (95% CI: -6.46 to 0.25); mesor, 92.43; acrophase, 05h20 (95% CI: 104.3 to 106.0). (C) Plasma cortisol: amplitude, 38.60 (95% CI: 20.78 to 56.42); mesor, 103.8; acrophase, 07h10 (95% CI: 100.5 to 101.5). (D) CSF cortisol: amplitude, 29.86 (95% CI: -38.91 to 20.82); mesor, 98.75; acrophase, 10h10 (95% CI: 102.1 to 103.4).

**Table 1.** Participant demographics.

**Table 1.** Participant demographics.

Number of participants	8
Age, mean	63.25 (57-69)
Body Mass Index, mean	24.0 (20.5-26.3)
Gender (Male) (%)	100 %
hsCRP (mg/L) at baseline	2.17 (0.16-9.92 )
hsCRP (mg/L) at last measure, mean (SD)	1.51 (0.27-5.16)



